

REQUEST FOR RECONSIDERATION AFTER FINAL	Application #	10/550,516
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	First Inventor	STRÖMBLAD, Staffan
	Art Unit	1612
	Examiner	PACKARD, Benjamin J.
	Docket #	P07900US01/BAS

Commissioner for Patents
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S I R:

In response to the Office Action dated August 18, 2009, Applicants submit the following Request for Reconsideration.

Claims 1-11 are pending in the present application. Applicants respectfully request that the rejection to the claims be reconsidered and the application found to be in condition for allowance based on the discussion which follows.

Claims 3-5 were rejected under 35 U.S.C. § 103(a) as being obvious over Bykov, et al. (hereinafter "Bykov"), in view of Hartmann, et al. (hereinafter "Hartmann"). In the rejection, it was asserted that Bykov appears to use the term "mutant" as synonymous with the term "defective". Further, the Examiner asserts that he interprets the term "mutant" in Bykov to broadly include inactive wild p53, alleging that an inactive conformational form creates a "defect" which inhibits the binding of p53 to DNA.

As an initial comment, Applicants gratefully appreciate the Examiner conducting a telephonic interview on October 17, 2009, discussing the uniqueness of treating a subpopulation of cancer patients, namely, patients having malignant melanoma cells

producing inactive wild-type p53, a new wild-type form of p53, previously not known and, thus, it would not have been obvious to treat this new subpopulation using the claimed method. In accordance with that Interview, Applicants present the following additional evidence of non-obviousness to treat a new and previously unknown subpopulation of malignant melanoma, based on a previously unknown form of wild-type p53, namely, inactive wild-type p53.

Contrary to the asserted rationale for maintaining the obviousness-type rejection, Bykov uses the term “mutant” in a manner completely different than the present specification with regard to “inactive wild-type p53” (see below, for further discussion). Further, inactive wild-type p53 is not present in all malignant melanoma cells. Therefore, novelty, in part, of the present invention lies in administering compounds of formula I only to patients which have inactive wild-type p53, i.e., a subpopulation of cancer patients. Absent knowing of a subpopulation of patients having a specific form of malignant melanoma cells which produce inactive wild-type p53, one of ordinary skill in the art would not have been led to administer the claimed compounds of formula I, as is discussed thoroughly in the Remarks to the May 11, 2009, Amendment, its contemporaneously filed Strömblad Declaration with supporting references, and as further discussed in additional discussion to follow.

In response to the assertion that Bykov (page 9, line 29) (as the published PCT application, WO02/24692) appears to use the term “mutant” as synonymous with the term “defect”, Applicants respectfully submit that this interpretation is incorrect. Bykov, by inserting the word “defect” after the word “mutant”, distinguishes a mutant and defective p53 from a mutant but non-defective p53. Moreover, it is well known to a

person of ordinary skill in the art that not every mutation gives rise to a defective protein. This is why Bykov specifies that the mutant p53 is defective. However, it must be emphasized that Bykov does not in any way teach or suggest that a wild-type p53 protein could be inactive.

Furthermore, it is relevant to note that the applicant/assignee of Bykov is also the same assignee as the present application. Moreover, one of the co-inventors, Dr. Wiman of Bykov, confirms that the inference alleged by the Examiner is erroneous and the use of the term “mutant (defect)” (Bykov, page 9, line 29, PCT 2002/24692) does not correspond to the claimed inactive wild-type p53. Should it be necessary, the present Applicants can submit a Rule 132 Declaration from Dr. Wiman confirming that the intention of the inventors of Bykov, as well as what one of ordinary skill in the art would have understood from the Bykov reference, would not in any way lead one of ordinary skill in the art to believe that the disclosed term “mutant (defect)” is synonymous with the presently claimed term of “inactive wild-type p53”, as defined in the present specification, and as claimed.

Based on the foregoing, contrary to the assertion of the Examiner, Bykov does not use the term “mutant” as synonymous with the term “defect”, let alone the claimed term “inactive wild-type p53”.

Further, in response to the Examiner’s interpretation that the term “mutant” broadly includes “inactive wild-type p53”, such an interpretation is incorrect and is inconsistent with how the term “mutant” would be understood by one of ordinary skill in the art. According to general principles, a wild-type protein is one having no mutation in its amino acid sequence. Therefore, to allege that “mutant” includes “inactive wild-type

p53” is completely incorrect and nonsensical as a protein **cannot** be ***both*** mutant and wild-type.

Furthermore, while a person of ordinary skill in the art at the time of the present invention knew that a mutant p53 could either be active or (more or less) inactive, i.e., more or less defective, it was completely unexpected that a wild-type p53 also could be inactive. Therefore, contrary to the obviousness-type rejection, it would not have been obvious to a person of ordinary skill in the art that a wild-type p53 could behave like mutant p53.

Moreover, one of ordinary skill in the art would not have been led to treat melanoma cells, let alone malignant melanoma cells having an inactive wild-type p53, using the claimed method from Bykov, in view of Hartmann. Both Bykov and Hartmann are directed to methods for treating specific cancers which were previously identified as having “mutant p53”. Accordingly, both methods specifically are directed to methods which are directed to treating mutant p53. Neither reference identifies any specific cancer associated with the claimed “inactive wild-type p53”. Furthermore, Hartmann actually teaches that, surprisingly, mutant p53 is not found in any significant amount and, therefore, one skilled in the art would not be led to use a method which targets mutant p53 to treat malignant melanoma. Therefore, absent knowing that there would have been any benefit from treating a specific group or subgroup of cancers, namely, malignant melanoma cells which have an inactive wild-type p53, one of ordinary skill in the art would not have been led to use the claimed compound of formula I to treat a previously unknown subgroup of cancer cells, namely, malignant melanoma cells having inactive wild-type p53, with the claimed compound. As discussed in great detail

previously, including the Remarks to the May 11, 2009, Amendment, Bykov is directed to treating mutant p53 and Hartmann discloses that mutant p53 is present in some cancers, but not in any significant amount in malignant melanoma cells. Neither reference teaches or in any way makes obvious that a new subgroup of cancer cells exist which have inactive wild-type p53, let alone malignant melanoma cells with this inactive wild-type p53 can be treated using the claimed method.

Further, as disclosed in the Background section in the present specification, it was previously known that p53 is mutated in most human tumors, yet wild-type p53 is dominant in malignant melanoma (see, e.g., present specification, page 1, lines 17-23, also citing Hartmann). What was not previously known is that the wild-type p53 in malignant melanoma cells can be in a newly discovered inactive form. Thus, while it may have been obvious to one of ordinary skill in the art to treat known tumors which produce mutant p53, it would not have been obvious to one of ordinary skill in the art to treat tumors which have inactive wild-type p53.

In response to the Examiner's statement that the recognition that PRIMA-1 not only activates mutant p53, but also activates inactive wild-type p53, does not render the instant claims patentable over the prior art, where the same agent (e.g., PRIMA-1) is administered to the same population (patients with malignant melanoma), it must be emphasized that prior to the present invention, malignant melanoma was not considered as being treatable with compounds such as PRIMA-1, directed to restoring the activity of malfunctioning p53 (i.e., mutant p53). This is due to the simple reason that it was known that malignant melanoma only rarely included mutated p53, see, for example, Hartmann and the attached article by C. C. Harris, 1996 (hereinafter "Harris"),

attached hereto in Appendix A), where it was noted that p53 tumors suppressor gene is frequently mutated in the human cancer, including squamous cell carcinoma and basal cell carcinoma of the skin, but is rarely mutated in skin melanoma. Therefore, prior to the present invention, the patient population was generally recognized as treatable with compounds directed to restoring inactive malfunctioning p53, such as PRIMA-1, which did not include patients with malignant melanoma. Therefore, contrary to the assertions of the Examiner, the present invention does not result in a situation where “the same agent is administered to the same population”.

In addition, in response to the Examiner’s assertion that one of ordinary skill in the art would expect, when treating a large population of patients with “mutant” p53, that a subpopulation will have the instantly recognized “inactive wild p53”, Applicants respectfully submit that such an inference is incorrect. In malignant melanoma patients, a large population of patients have wild-type p53. This was well-known to one of ordinary skill in the art at the time of the present application (see, e.g., Harris Appendix A). On the other hand, it is also well-known that tumor cells have one of the p53 alleles mutated generally, will not have the other allele as a wild-type. Rather, what was understood at the time of the priority date of the present application, is that it was well-known in the prior art that, in most tumor cells where one of the p53 alleles is mutated, the other one is deleted. See, e.g., Baker, et al. (1989) (hereinafter “Baker”) and Nigro, et al. (1989) (hereinafter “Nigro”), herein attached as Appendices B and C, respectfully.

Baker, in the abstract, lines 8-13, notes that in two examined colorectal tumors, one p53 allele is deleted and the other one is mutated. At page 219, center column,

lines 5-8, and right column, lines 6-9, the results from the sequence analysis of the remaining p53 allele in the two tumors are disclosed, colon one has a mutation in codon 143 and the other one in codon 175. Confirming results are shown in Figures 3 and 4 on page 219.

In Nigro, analysis of a larger variety of tumors is presented. The results are compiled in Table 1, page 707. In the abstract, lines 20-21, the authors conclude that “most tumors with such allelic deletions contain p53 point mutations resulting in amino acid substitutions”. In other words, in tumor cells where one allele is deleted, the other one is generally mutated. The authors further note that such mutations also occur in tumors retaining bulk alleles (Nigro, lines 21-24). From the data in Table 1, it is noted that out of 22 tumors analyzed, only one (tumor number 17) shows no p53 mutation/deletion at all.

In stark contrast to the p53 mutation rate in malignant melanoma cells, see, Hartmann, e.g., the discussion at page 315, where it is noted that mutations in the p53 gene were found in only 8 out of 191 primary and metastasized melanomas and of 105 primary melanomas, only one had alterations in the p53 gene. Therefore, at the time of the present invention, it was generally recognized that the population of cancer patients with mutant p53 generally do not include patients suffering from malignant melanoma and that malignant melanoma tumors generally are not linked to mutation in the p53 gene.

In conclusion, a physician treating a patient suffering from a potentially fatal disease with high mortality, such as malignant melanoma, generally will not prescribe a treatment that is considered not efficacious. Therefore, a person of ordinary skill in the

art, based on knowledge in the art at the time of the present invention, would not have been led or in any way would consider treating malignant melanoma using the claimed method as such a method would not have been considered efficacious at the time of the present invention. Accordingly, prior to the present invention, patients suffering from malignant melanoma would not have received treatment directed to restoring the function to p53 for the simple reason that this mode of treatment was not considered to have had any effect in this specific cancer population (i.e., the one claimed). By the efforts of the present inventors, the patient population of persons suffering from malignant melanoma will be able to benefit from a treatment prior to the present invention not considered to have been useful. Therefore, it is now expected that a high mortality and suffering linked to this disease can be reduced.

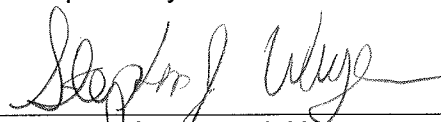
Based on the foregoing, Applicants respectfully request that the rejection to claims 3-5 under 35 U.S.C. § 103(a) be withdrawn.

In view of the foregoing, Applicants respectfully submit that the present application is in condition for allowance.

Respectfully submitted,

Date: November 18, 2009

Signed By
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APPENDIX A

p53: At the Crossroads of Molecular Carcinogenesis and Molecular Epidemiology

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The p53 tumor suppressor gene is frequently mutated in human cancer, including squamous cell carcinoma and basal cell carcinoma of the skin, but is rarely mutated in skin melanoma. The p53 mutation spectrum provides insight into both the etiology of hu-

man cancer and the functional regions of the encoded protein that lead to clonal expansion of the p53 mutant cell. *Journal of Investigative Dermatology Symposium Proceedings* 1:115-118, 1996

CANCER SUSCEPTIBILITY GENES

The crucial differences between normal and cancer cells stem from discrete changes in specific genes controlling proliferation and tissue homeostasis. More than 100 such cancer-related genes have been discovered, several of which are implicated in the natural history of human cancer because they are consistently found mutated in tumors. The p53 tumor suppressor gene is the most striking example because it is mutated in about half of almost all cancer types arising from a wide spectrum of tissues. Other tumor suppressor genes important in human oncology, e.g., APC, WT1, p16^{INK4}, or NF1, may have a more limited distribution (Table I), but given the variety of hereditary cancers and allelic deletions found in human cancers, additional tumor suppressor genes should be identified in the future, some of which may also have a conspicuous role in carcinogenesis.

Tumor suppressor genes are vulnerable sites for critical DNA damage because they normally function as physiologic barriers against clonal expansion or genomic mutability, and are able to hinder growth and metastasis of cells driven to uncontrolled proliferation by oncogenes. Loss of tumor suppressor function can occur by damage to the genome through mutation, chromosomal rearrangement and nondisjunction, gene conversion, imprinting, or mitotic recombination. Tumor suppressor activity can also be neutralized by interaction with other cellular proteins or with viral oncoproteins. Comprehensive reviews of this rapidly advancing field of molecular carcinogenesis are available (Bishop, 1991; Harris, 1991; Weinberg, 1991).

p53 TUMOR SUPPRESSOR GENE

The p53 suppressor gene is the most prominent example because it is mutated in about half of human cancer cases (Hollstein *et al.*, 1991; Greenblatt *et al.*, 1994). Although the retinoblastoma and APC tumor suppressor genes are most commonly inactivated by nonsense mutations, which cause the protein to be truncated or unstable, about 80% of p53 mutations are missense mutations, which change the identity of an amino acid. Changing amino acids in this way can alter the protein conformation and increase the stability of p53; it can also alter sequence-specific DNA binding and transcription factor activity of p53 (Vogelstein and Kinzler, 1992).

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One explanation for the high frequency of p53 mutation is that the missense class of mutations can cause both a loss of tumor suppressor function and a gain of oncogenic function by changing the repertoire of genes whose expression is controlled by this transcription factor (Lane and Benichou, 1990; Dittmer *et al.*, 1993). The central role of p53 in multistage carcinogenesis places it at the intellectual crossroads of molecular carcinogenesis, molecular epidemiology of human cancer, and cancer risk assessment.

p53 participates in many cellular functions: cell cycle control, DNA repair, differentiation, genomic plasticity, and programmed cell death (Lane and Benichou, 1990; Levine *et al.*, 1991; Greenblatt *et al.*, 1994). In mammalian cells, p53 is one component of the DNA damage response pathway (Fig 1). Some of these normal cellular functions of p53 can be modulated and sometimes inhibited by interactions with either cellular proteins, e.g., mdm2, or oncoviral proteins, e.g., hepatitis B virus X protein, of certain DNA viruses. p53 is clearly a component in a biochemical pathway or pathways central to human carcinogenesis, and p53 mutations provide a selective advantage for clonal expansion of preneoplastic and neoplastic cells.

The spectrum of mutations in p53 induced in human cancer can help identify particular carcinogens and define the biochemical mechanisms responsible for the genetic lesions in DNA that cause human cancer. The frequency and type of p53 mutations can also act as a molecular dosimeter of carcinogen exposure and thereby provide information about the molecular epidemiology of human cancer risk. The p53 gene is well suited for this form of molecular archaeology. The majority of mutations in p53 are in the hydrophobic midregion of the protein (Fig 2) (Greenblatt *et al.*, 1994). The function of the p53 protein as a transcription factor is exquisitely sensitive to conformational changes in this region that result from amino acid substitutions (Chen *et al.*, 1994), and p53 binding to other cellular and oncoviral proteins can easily be disrupted by mutations in these regions.

How can p53 mutation spectra lead to identification of the carcinogens that caused a particular tumor? Different carcinogens seem to cause different characteristic mutations. Exposure to one common carcinogen, ultraviolet light, is correlated with transition mutations at dipyrimidine sites (Fig 3) (Brash *et al.*, 1991). Accumulation of nuclear p53 and p53 mutations are found in both preinvasive and invasive skin tumors (Rady *et al.*, 1992; Stephenson *et al.*, 1992; Nagano *et al.*, 1993; Kerschmann *et al.*, 1994; Bito *et al.*, 1995; Borkowski *et al.*, 1996). Dietary aflatoxin B₁ exposure is

Table I. Examples of Tumor Suppressor Genes Involved in Human Cancers^a

Tumor Suppressor Gene	Chromosomal Locus	Location/Proposed Function	Somatic Mutations		Inherited Mutations		
			Major Types	Examples of Neoplasms	Syndrome	Heterozygote Carrier Rate Per 10 ⁵ Births	Typical Neoplasms
p53	17p13.1	Nuclear/transcription factor	Missense	Most human cancer types examined to date	Li Fraumeni	~2	Carcinomas of breast and adrenal cortex; sarcomas; leukemias; brain tumors
RB1	13q14	Nuclear/transcription modifier	Deletion and nonsense	Retinoblastoma; osteosarcoma; carcinomas of the breast, prostate, bladder, and lung	Retinoblastoma	~2	Retinoblastoma; osteosarcoma
APC	5q21	Cytoplasmic/unknown	Deletion and nonsense	Carcinoma of the colon, stomach, and pancreas	Familial adenomatous polyposis	~10	Carcinomas of colon, thyroid, and stomach
ATM	11q22	Unknown/kinase	Deletion	Unknown	Ataxia/telangiectasia	~2	Leukemia; lymphoma
WT1	11p13	Nuclear/transcription factor	Missense	Wilms tumor	Wilms tumor	~0.5-1	Wilms tumor
NF1	17q11	Cytoplasmic/GTPase-activating protein	Deletion	Schwannoma	Neurofibromatosis type 1	~30	Neural tumors
NF2	22q	Cytoplasmic/cytoskeletal membrane linkage	Deletion and nonsense	Schwannomas and meningiomas	Neurofibromatosis type 2	~3	Central schwannomas and meningiomas
p16 ^{INK4a}	9p21	Nuclear cyclin-dependent kinase inhibitor	Deletion and nonsense	Mesothelioma; pancreas; melanoma; glioblastomas	Familial melanoma	?	Melanoma
VHL	3p25	Nuclear/adaptor	Deletion ^b	Unknown	von Hippel-Lindau	~3	Hemangioblastoma and renal cell carcinoma

^a Reviewed in Harris and Hollstein, 1993; Savitsky *et al.*, 1995.^b Downregulated expression controlled by epigenetic mechanism (DNA methylation) (Herman *et al.*, 1994).

correlated with G:C to T:A transversions that lead to a serine substitution at residue 249 of p53 in hepatocellular carcinoma (Bressan *et al.*, 1991; Ito *et al.*, 1991). Exposure to cigarette smoke is correlated with G:C to T:A transversions in lung carcinomas (Takeshima *et al.*, 1993).

How these mutations arise can be further tested in the laboratory. For example, the predominant base changes in p53 found in lung cancers (G:C to T:A transversions) and skin carcinomas (C:G to T:A transitions) suggest that the causal lesion likely occurred on the nontranscribed strand, a finding that is consistent with the preferential repair after damage of the transcribed strand of active genes

(Bohr, 1991). Benzo[a]pyrene, a carcinogen in tobacco smoke, forms DNA adducts that are repaired more slowly when present on the nontranscribed strand than on the transcribed strand of the hypoxanthine (guanine) phosphoribosyl transferase gene (Chen *et al.*, 1992), and ultraviolet light-induced cross-links of dipyrimidines in the nontranscribed DNA strand of the p53 gene also are repaired more slowly than in the transcribed strand (Evans *et al.*, 1993). Because DNA repair rates can be sequence dependent (Tornaietti and Pfeiffer, 1994), the p53 mutation spectrum could be influenced by both the type and location of the promutagenic lesion. Transcription-repair coupling factors, e.g., the product of the *mfd* gene, have been identified recently and provide a mechanistic underpinning for strand-specific repair (Buratowski, 1993; Schaeffer *et al.*, 1993; Selby and Sancar, 1993). The p53 protein binds to XPB and XPD DNA helicases in the TFIIH complex and modulates their function in nucleotide excision repair (Wang *et al.*, 1995). Another example comes from areas of China and Mozambique with a high incidence of liver cancer. The high frequency of G:C to T:A transversions in human hepatocellular carcinomas in these regions could be due to the high mutability of the third base of codon 249 by aflatoxin B₁ or a selective growth advantage of hepatocyte clones carrying this specific p53 mutant in liver chronically infected with hepatitis B virus. Indeed, the third base of codon 249 in a human liver cell line exposed to aflatoxin B₁ is preferentially mutated (Aguilar *et al.*, 1993), and transfected 249^{mut} p53 mutant enhances the growth rate of the p53 null hepatocellular carcinoma cell line, Hep3B (Ponchel *et al.*, 1994). Other p53 codons show a lower frequency of G:C to T:A, G:C to A:T, and G:C to C:G mutations, which suggests that both preferential mutability and clonal selection are involved in human hepatocellular carcinogenesis.

The p53 mutational spectra also can indicate that a particular

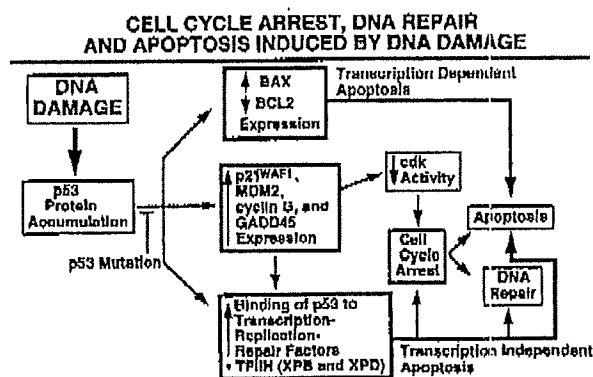


Figure 1. DNA damage leads to p53 accumulation and subsequent changes in gene expression and protein-protein interactions.

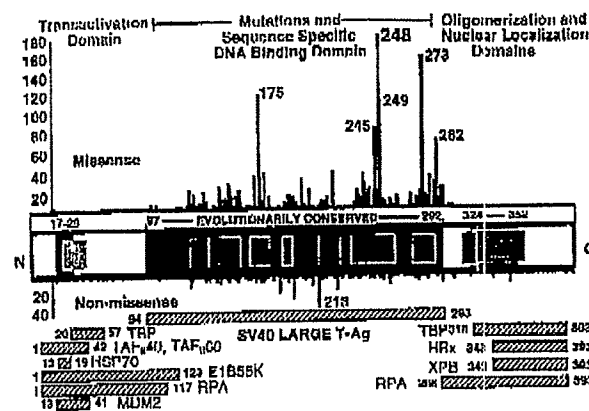


Figure 2. Schematic representation of the p53 molecule. The p53 protein consists of 393 amino acids with functional domains, evolutionarily conserved domains, and regions designated as mutational hot spots. Functional domains include the transactivation region (amino acids 20-42; light gray area), sequence-specific DNA binding region (amino acids 100-293), nuclear localization sequence (amino acids 316-325; dark gray area), and oligomerization region (amino acids 319-360; dark gray horizontal striped area). Cellular or uncoupled proteins bind to specific areas of the p53 protein. Evolutionarily conserved domains (amino acids 17-29, 97-292, and 324-352; black area) were determined using the MACAW program. Seven mutational hot spot regions within the large conserved domain are identified (amino acids 130-142, 151-164, 171-181, 193-200, 213-223, 234-258, and 270-286; medium gray areas). Vertical lines show the schematic, missense mutations; lines below schematic, non-missense mutations. The majority of missense mutations are in the conserved hydrophobic nucleation, whereas non-missense mutations (nonsense, frameshift, splicing, and silent) are distributed throughout the protein, determined primarily by sequence context.

cancer did not result from an environmental carcinogen but instead was caused by endogenous mutagenesis. The high frequency of C to T transitions at CpG dinucleotides in colon carcinomas (Hollstein *et al.*, 1991) is consistent with mutagenesis by endogenous deamination mechanisms. A transition of C to T would be generated by spontaneous deamination of 5-methylcytosine (Rideout 3d *et al.*, 1990) or by enzymatic deamination of cytosine by DNA (cytosine-5)-methyl transferase when S-adenosylmethionine is in limiting concentration (or by both mechanisms) (Shen *et al.*, 1992). Because oxygen radicals enhance the rate of deamination of deoxynucleotides (Wink *et al.*, 1991; Nguyen *et al.*, 1992), chronic inflammation and nitric oxide generated by nitric oxide synthases

p53 MUTATIONAL SPECTRUM IN HUMAN SKIN CANCER

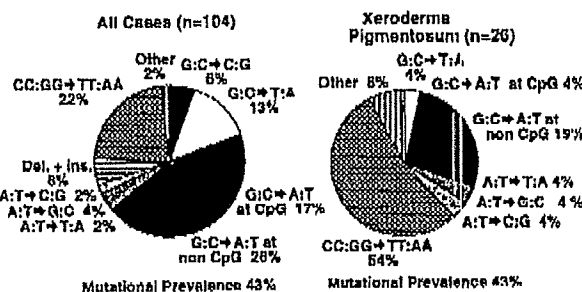


Figure 3. A comparison of the p53 mutational spectra in skin carcinomas from normal versus xeroderma pigmentosum group C donors.

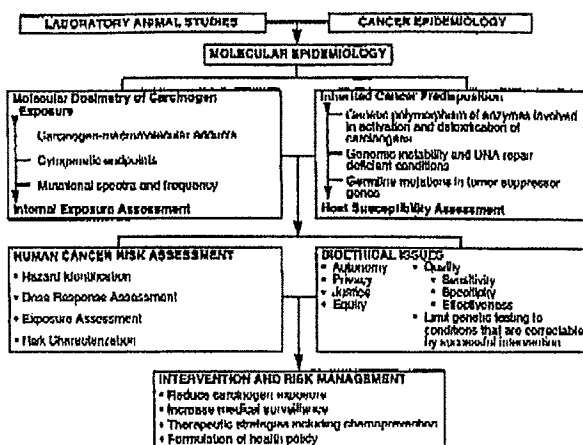


Figure 4. Human cancer risk assessment and bioethical issues associated with molecular epidemiology and human cancer.

may explain why rats that inhale particulate materials that cause inflammation, but do not lack act directly on DNA, have a high incidence of lung cancer.

Mutations in p53 can also reveal that an individual has an increased susceptibility to cancer owing to inheritance of a germline mutation, a concept first proposed for the retinoblastoma (Rb) tumor suppressor gene (Knudson Jr, 1971). Germline p53 mutations are missense and occur frequently in cancer-prone individuals with Li-Fraumeni syndrome (Malkin *et al.*, 1990). Laboratory animals with either a mutant p53 transgene or a deleted p53 gene, i.e., homozygous or heterozygous "gene knockouts," also are particularly susceptible to cancer (Lavigne *et al.*, 1989; Donehower *et al.*, 1992). These mutations in p53 are associated with instability in the rest of the genome (Hartwell, 1992). Such instability could generate multiple genetic alterations leading to cancer. Indeed, genomic instability (including gene amplification) increases in frequency in cells that lack a normal p53 gene (Livingstone *et al.*, 1992; Yin *et al.*, 1992). Furthermore, loss of the wild-type alleles of the p53 gene abrogates DNA damage-induced delay of the cell cycle in G1 (Kastan *et al.*, 1992). DNA repair of certain promutagenic lesions can proceed before DNA synthesis in S phase. Loss time for repair would increase the frequency of mutations. Because p53 is an integral component in one pathway of programmed cell death (apoptosis) induced by DNA-damaging chemotherapeutic drugs or ionizing radiation (Clarke *et al.*, 1993; Lowe *et al.*, 1993), inactivation of p53 could increase both the pool of proliferating cells and the probability of their neoplastic transformation by inhibition of programmed cell death.

MOLECULAR EPIDEMIOLOGY OF CANCER

Such progress in the fields of molecular carcinogenesis and molecular epidemiology increases our ability to assess cancer risk accurately (Fig 4). Cancer risk assessment, a highly visible discipline in public health, has historically relied on classic epidemiology, from chronic exposure of rodents to potential carcinogens and the mathematical modeling of these findings. The field has been forced to steer a prudent course of conservative risk assessment because of limited knowledge of the complex pathobiologic processes during carcinogenesis. Differences in the metabolism of carcinogens, different DNA repair capacities, variable genomic stability among animal species, and variation among individuals with inherited cancer predisposition have made definitive analysis of cancer risk almost impossible (Harris, 1991; Barrett and Wiseman, 1992). Because regulatory decisions based on cancer risk assessments have significant public health and economic consequences, the scientific

basis of risk assessment continues to be, and should remain, actively investigated (National Research Council, 1994).

Many questions remain. Are the pathways of molecular carcinogenesis similar in rodents and humans? Because the time to develop cancer is generally shorter in rodents than in humans, is the apparent interspecies difference due to the number of genetic and epigenetic events required for malignant progression or to the rate of transit between the events? Is the more frequent mutation of the *ras* proto-oncogenes in rodent cancer as compared with human cancer a reflection of a pathway that is parallel and equivalent to the p53 pathway in human carcinogenesis? Are the selective pressures for clonal expansion of preneoplastic and neoplastic cells in human carcinogenesis similar to those in animal models?

Investigations of the p53 tumor suppressor gene are an example of the recent progress in molecular aspects of cancer research. A better understanding of molecular carcinogenesis and molecular epidemiology will eventually decrease the qualitative and quantitative uncertainties associated with the current state of cancer risk assessment and will improve public health decisions concerning cancer hazards. Indeed, determination of the type and number of mutations in p53 and other cancer-related genes in tissues from "healthy" people may allow identification of those at increased cancer risk and their consequent protection by preventive measures.

The editorial and graphic assistance of Dorothea Dudek is appreciated.

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APPENDIX B

Since the phenomenon of self-MHC restriction was first described, positive selection had been inferred to be the mechanism, but the evidence was always indirect. Now positive selection has been formally demonstrated. The mechanism involves the interaction of TCR chains, accessory molecules, and MHC during thymus development.

Note added in proof: Recently, positive selection has been proposed to explain the correlation between levels of T cells bearing $V_{\beta}6^{+}$ TCR and MHC class II I-E molecules in MHC congenic and F_1 mice (24).

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Chromosome 17 Deletions and p53 Gene Mutations in Colorectal Carcinomas

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Previous studies have demonstrated that allelic deletions of the short arm of chromosome 17 occur in over 75% of colorectal carcinomas. Twenty chromosome 17p markers were used to localize the common region of deletion in these tumors to a region contained within bands 17p12 to 17p13.3. This region contains the gene for the transformation-associated protein p53. Southern and Northern blot hybridization experiments provided no evidence for gross alterations of the p53 gene or surrounding sequences. As a more rigorous test of the possibility that p53 was a target of the deletions, the p53 coding regions from two tumors were analyzed; these two tumors, like most colorectal carcinomas, had allelic deletions of chromosome 17p and expressed considerable amounts of p53 messenger RNA from the remaining allele. The remaining p53 allele was mutated in both tumors, with an alanine substituted for valine at codon 143 of one tumor and a histidine substituted for arginine at codon 175 of the second tumor. Both mutations occurred in a highly conserved region of the p53 gene that was previously found to be mutated in murine p53 oncogenes. The data suggest that p53 gene mutations may be involved in colorectal neoplasia, perhaps through inactivation of a tumor suppressor function of the wild-type p53 gene.

RECENT STUDIES HAVE ELUCIDATED several genetic alterations that occur during the development of colorectal tumors (1–3), the most common of which are deletions of the short arm of chromosome 17. While some genetic alterations, such as RAS mutations, appear to occur relatively early during colorectal tumor development, chromosome 17p deletions are often late events associated with the transition from the benign (adenomatous) to the malignant (carcinomatous) state (1). Because carcinomas are often lethal, while the precursor adenomas are uniformly curable, the delineation of the molecular events mediating this transition are of considerable importance. The occurrence of allelic deletions of chromosome 17p in a wide variety of cancers besides those of the colon, including those of the breast and lung, further emphasizes the importance of identi-

fying genes on 17p that are involved in the neoplastic process (4).

Our approach to this identification was based on first defining a small region of chromosome 17p that is commonly lost in different colorectal carcinomas. Twenty DNA probes detecting restriction fragment length polymorphisms (RFLPs) on chromosome 17p were used to examine the patterns of allelic losses in colorectal tumors. These probes have been mapped to seven discrete regions of 17p on the basis of their hybridization to human-rodent somatic cell hybrids containing parts of chromosome 17p (5). DNA was obtained from 58 carcinoma specimens and compared to DNA from adjacent normal colonic mucosa. Allelic losses were scored if either of the two alleles present in the normal cells was absent in the DNA from the tumor cells. Allelic deletions can be difficult to detect in DNA prepared from whole tumors because most solid tumors contain a significant number of non-neoplastic stromal and inflammatory cells. For this reason, regions of tumors containing a high proportion of neoplastic cells were isolated, and DNA was prepared from cryostat sections of these regions as described (6).

The two parental alleles could be distinguished in the normal mucosa of each patient by at least 5 of the 20 RFLP markers (the "informative" markers for each case). Seventy-seven percent of the tumors exhibited allelic losses of at least three markers. Studies of eight tumors that retained heterozygosity for some but not all markers on

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chromosome 17p enabled us to define a common region of deletion. For example, the tumor from patient S51 had retained both parental alleles of three markers from the distal region of 17p, but had lost one allele of all the more proximal markers that were informative (Fig. 1, A to C, and Fig. 2). This implied that the target of the allelic loss in this tumor was proximal to the three retained markers. The tumor from patient S103 had retained both parental alleles at all informative loci proximal to EW505, but had allelic deletions of several more distal markers (Fig. 1, D to F, and Fig. 2). The combined data depicted in Fig. 2 indicated that the smallest common region of deletion extended between markers within band 17p12 to those within band 17p13.3. This localization is based on the assumption that the same 17p locus was the target of deletion in all of the tumors.

Allelic deletions are thought to signal the presence of a tumor suppressor gene within the affected region of the chromosome (7). The tumor suppressor gene represents the critical gene ("target") of the deletion event. When both the maternal and paternal copies of such a gene are inactivated, suppression may be relieved and abnormal proliferation ensue. One scenario for the functional loss of tumor suppressor genes involves the inactivation of one allele through an inherited or somatic mutation (7). This inactivation is accompanied by loss of the remaining normal allele through a gross chromosomal change such as loss of a whole chromosome. An obligatory feature of this scenario is that the suppressor gene allele remaining in the tumor should contain a mutation.

The gene encoding p53 has been previously localized to region D₁ of chromosome 17p (5), which is within the common region of deletion observed in colorectal tumors (Fig. 2). As the p53 gene product has been implicated in the process of neoplastic transformation (8), we attempted to determine whether this gene might be a target of the deletions in colorectal tumors.

First, p53 cDNA probes detecting exons spread over 20,000 bp [including all protein encoding exons (9)] were used to examine the DNA of 82 colorectal carcinomas (50 primary specimens and 32 cell lines) in Southern blotting experiments. No rearrangements of the p53 gene were observed in Eco RI or Bam HI digests, nor were deletions of both alleles seen (10). As p53 expression might be affected by gross genetic alterations further removed from p53 coding sequences, pulsed-field gel electrophoresis was used to examine large restriction fragments encompassing the p53 gene. The restriction endonucleases Eco RV, Pae R7I, Not I, and Sal I generated p53 gene-

Fig. 1. Allelic deletions on chromosome 17p. DNA from normal (N) and carcinoma (C) tissue of patients S51 and S103 was digested with restriction endonucleases and the fragments separated by electrophoresis. After transfer to nylon filters, the DNA was hybridized to radiolabeled probes. Autoradiographs of the washed filters are shown. The alleles designated "1" and "2" refer to the larger and smaller polymorphic alleles, respectively, present in the normal DNA samples. The probes used were (A) MCT35.2; (B) EW301; (C) YNH37.3; (D) YNZ22.1; (E) MCT35.1; and (F) EW505. Deletions of allele 1 can be seen in panels A and E; deletions of allele 2 in panels B and D. Areas of tumors containing a high proportion of neoplastic cells were identified histopathologically in cryostat sections, and 12- μ m-thick cryostat sections of these areas were used to prepare DNA (6). Grossly normal colonic mucosa adjacent to the tumors was obtained from each patient and used to prepare control DNA. DNA purification, restriction endonuclease digestion, electrophoresis, transfer, and hybridization were as described (1, 6). Taq I digestion was used for panels A, B, C, and F; Bam HI for panel D; and Msp I for panel E.

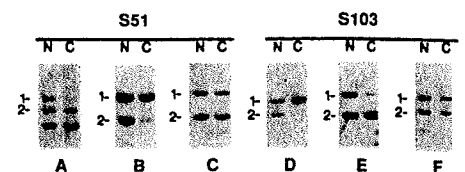
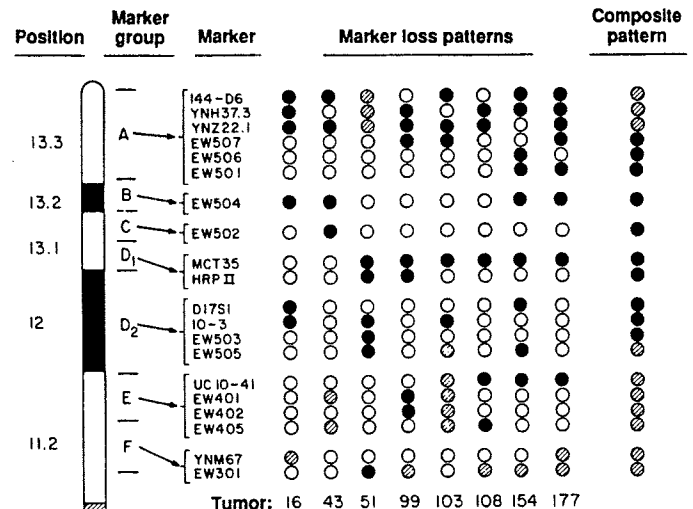


Fig. 2. Map of the common region of 17p deletion in colorectal tumors. Chromosomal positions of 20 markers from chromosome 17p are indicated. The markers were previously localized (5) to seven subchromosomal regions (A to F). Hybridization results for eight tumors are shown on the right, with patient identification numbers indicated at the bottom. For each of the 20 markers, a filled circle indicates that one parental allele was lost in the tumor; a cross-hatched circle indicates that both parental alleles were retained in the tumor; an open circle indicates that the marker was not informative (the patient's normal tissue was not heterozygous for the marker). The composite pattern (far right) assumes that there was only one target gene on chromosome 17p, so that markers for which heterozygosity was retained in any of the eight tumors would be outside the target gene locus. The region between probes YNZ22.1 and EW505 was deleted in every tumor in which markers in this region were informative.



containing fragments of 45 to 350 kb from the DNA of normal cells. No alterations were detected in the DNA from any of 21 colorectal tumor cell lines examined with each of these four enzymes (10).

We next considered the possibility that p53 gene inactivation could occur through interference with mRNA expression in the absence of gross changes in gene structure. To assess this possibility, we performed Northern blot experiments on RNA from 22 colorectal tumors (six primary tumors and 16 cell lines). The expression of p53 has been correlated with cellular growth and/or transformation (11); for this reason, other genes whose expression is similarly regulated were used as controls (12). The size of p53 mRNA was normal (2.8 kb) in all 22 tumors (10). Moreover, the relative abundance of p53 gene mRNA was usually at least as great in colorectal tumor cells as in normal colonic mucosa, confirming the results of Calabretta *et al.* (12). However, in

four tumors, relatively little expression of p53 mRNA was observed compared to that in the other tumors. This low level of expression of p53 was specific in that *c-myc*, histone H3, and phosphoglycerate kinase mRNA's were expressed in these four tumors at levels similar to those seen in other colorectal tumors and at least as high as in non-neoplastic colonic mucosa (10).

The absence of gross alterations in p53 gene structure and expression in most colorectal carcinomas did not exclude the presence of subtle alterations of the p53 gene in these cases. To test for such subtle alterations, a tumor was chosen that had an allelic deletion of chromosome 17p yet expressed significant quantities of p53 mRNA. A cDNA clone originating from the remaining p53 allele was isolated and sequenced to determine whether the gene product was abnormal.

For practical reasons, a nude mouse xenograft (Cx3) of a primary tumor was selected

for this test. Primary tumors contain non-neoplastic cells that could contribute p53 mRNA, while in xenografts the non-neoplastic cells (derived from the mouse) could not be the source of a human p53 cDNA clone. Cx3, like over 75% of colorectal carcinomas, had allelic deletions of several RFLP markers on chromosome 17 and expressed significant amounts of p53 mRNA (10).

A nearly full-length p53 cDNA was cloned from Cx3 mRNA by standard techniques (13). The clone extended 2567 nucleotides (nt) from position -198 relative to the translation initiation site to the poly-

adenosine tail. The clone was sequenced by the dideoxy chain-termination method and one nucleotide difference was identified in comparison with published p53 cDNA sequences (9). A transition from T to C had occurred within codon 143 (GTG to GCG), resulting in a change of the encoded amino acid from valine to alanine. To ensure that the sequence change was not an artifact of cDNA cloning, the polymerase chain reaction [PCR, (14)] was used to amplify a 111-bp sequence surrounding the presumptive mutation from genomic DNA of Cx3. Analysis of the PCR product was facilitated by the observation that the presumptive mutation created a new Hha I site (GCGC at nt 427 to 430). The 111-bp PCR product from tumor Cx3 was cleaved with Hha I to produce the expected 68- and 43-bp subfragments (Fig. 3, lanes 5 and 6). The 111-bp PCR product from the DNA of normal cells of the patient providing Cx3 was not cleaved with Hha I (Fig. 3, lanes 7 and 8), nor were the PCR products of 37 other DNA samples prepared from the normal tissues, primary colorectal tumors, or xenografts of other patients (examples in Fig. 3, lanes 1 to 4). Therefore, the valine to alanine substitution present in this tumor was the

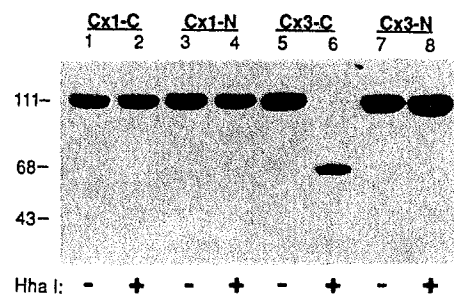


Fig. 3. Polymerase chain reaction analysis of p53 codon 143. A 111-bp fragment surrounding p53 codon 143 was amplified from genomic DNA by means of Taq polymerase (14). Half of the preparation was cleaved with Hha I (lanes marked "+"); the other half was not treated further (lanes marked "-"). After electrophoresis, PCR DNA fragments were detected by hybridization to a labeled p53 cDNA probe. The DNA samples used for PCR were derived from lanes 1 and 2, colorectal tumor (C) xenograft Cx1; lanes 3 and 4, normal (N) fibroblasts from the patient providing Cx1; lanes 5 and 6, colorectal tumor xenograft Cx3; lanes 7 and 8, normal fibroblasts from the patient providing Cx3. Only in tumor xenograft Cx3 (lane 6) did Hha I cleave the 111-bp fragment to the expected 68- and 43-bp subfragments (the 43-bp subfragment hybridized only weakly because of its small size). DNA was incubated in the presence of Taq polymerase with primer oligomers complementary to sequences 68 bp upstream and 43 bp downstream of codon 143. The upstream primer used was 5'-TTCCTC-TTCCTGCAGTACTCC-3'; all but six nucleotides of this primer were derived from the p53 intron 4 sequence determined by Buchman *et al.* (9). The downstream primer was 5'-GACGCGG-GTGCCGGGCGG-3'. After 35 cycles of denaturation (1 min, 93°), annealing (2 min, 55°) and elongation (2 min, 70°) amplified DNA fragments of 111 bp were generated. After electrophoresis, the 111-bp amplified fragments were eluted from a polyacrylamide gel and purified by extraction with phenol and chloroform. A small amount of a contaminating 73-bp PCR product was present in most of the eluates; the contaminant was not cleaved by Hha I, however, so that it did not interfere with the analysis. A portion of each of the purified DNA fragments was digested with Hha I, separated by electrophoresis on a nondenaturing polyacrylamide gel, and electrophoretically transferred to nylon filters. The fragments were hybridized with a radioactive probe generated from a 1.8-kb Xba I fragment of a p53 cDNA clone provided by D. Givol (9).

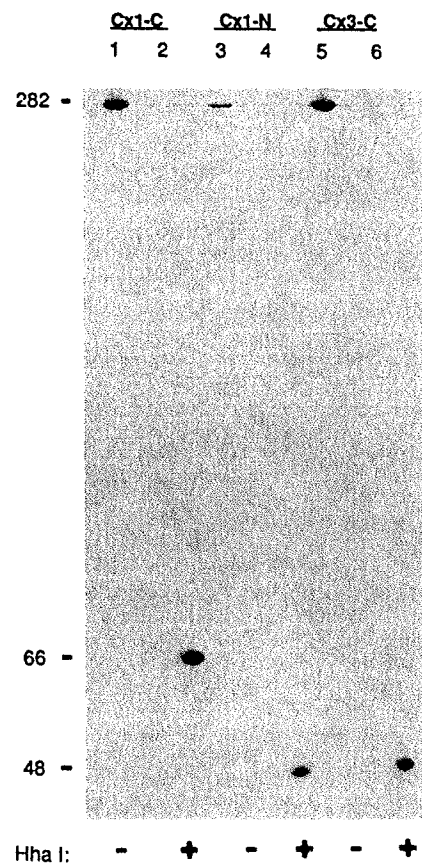


Fig. 4. Polymerase chain reaction analysis of p53 codon 175. A DNA fragment containing p53 codon 175 was amplified from genomic DNA by means of Taq polymerase and radioactively labeled at one end. A portion of the preparation was cleaved with Hha I (lanes marked "+"); another portion was not treated further (lanes marked "-"). The labeled fragments were then separated by electrophoresis and visualized through autoradiography. The DNA samples were derived from lanes 1 and 2, colorectal tumor (C) xenograft Cx1; lanes 3 and 4, normal (N) fibroblasts from the patient providing Cx1; lanes 5 and 6, colorectal tumor xenograft Cx3. A 48-bp Hha I fragment is produced if codon 175 is wild-type; a 66-bp Hha I fragment (present only in tumor Cx1) is produced if codon 175 is mutated. PCR was used to amplify a 319-bp fragment containing intron 5 and surrounding exon sequences. The upstream primer was the same as used for primer set 3 (15) and the downstream primer was 5'-CGGAATTCAGGCGGCTCATA-GGGC-3'; PCR was performed as described in the legend to Fig. 3. After electrophoresis through a 2% agarose gel, the 319-bp fragment was purified by binding to glass beads (30). The DNA fragments were cleaved with Sty I at nt 477 and end-labeled by fill-in with the Klenow fragment of DNA polymerase I and ³²P-labeled dCTP. After electrophoresis of the reaction mixture through a non-denaturing polyacrylamide gel, the 282-bp Sty I fragment (nt 477 to 758), labeled at the proximal end and containing codon 175, was eluted and purified by extraction with phenol and chloroform. A portion of the eluted DNA was cleaved with Hha I and the fragments separated by electrophoresis on a 6% sequencing gel. Hha I digestion of the 282-bp Sty I fragment produces a labeled 48-bp fragment (comprising nt 477 to 524) if codon 175 is wild type. If codon 175 is mutated, a labeled 66-bp fragment (comprising nt 477 to 542) is produced by Hha I as a result of cleavage at the first Hha I site downstream of codon 175.

result of a specific point mutation not present in the germline of the patient.

A similar strategy was applied to the analysis of the remaining p53 allele of a colorectal tumor (Cx1) from another patient (15). A single point mutation was identified, which resulted in the substitution of histidine for arginine at codon 175 (transition from CGC to CAC). To ensure that the sequence change represented a mutation rather than a sequence polymorphism, PCR was used to amplify a fragment containing codon 175 from the genomic DNA of tumor Cx1 and normal cells. The presumptive mutation abolished the Hha I site normally present at codon 175 (GCGC at nt 522 to 525). Thus, Hha I cleavage of the PCR products from DNA of the normal cells of the patient providing Cx1 (Fig. 4, lanes 3 and 4) or from the tumor of another patient (Fig. 4, lanes 5 and 6) produced only the 48-bp product expected if codon 175 was wild type (see legend to Fig. 4). In contrast, the PCR product from tumor Cx1 was not cleaved at nt 524 (corresponding to codon 175) and exhibited only a larger 66-bp fragment resulting from cleavage at a normal downstream Hha I site at nt 542.

Thus, most colorectal tumors contained

deletions of the region containing the p53 gene, and the p53 gene was mutated in both tumors subjected to detailed analysis. There are many potential explanations for these findings: (i) p53 is not involved in colorectal tumorigenesis and the p53 abnormalities identified were coincidental epiphenomena, (ii) p53 is a target of the 17p deletions in all colorectal tumors, (iii) p53 is a target in some tumors, such as Cx3 and Cx1, but a different chromosome 17p gene is the target in other tumors, or (iv) p53 is not a target of deletion in any tumor, but mutations of p53 can provide a selective growth advantage complementing that derived from an unidentified tumor suppressor gene on 17p.

We cannot differentiate among these possibilities at present, but feel that the first explanation is unlikely for several reasons. Most importantly, the mutations in Cx1 and Cx3 were clonal, that is, they occurred in all of the neoplastic cells of the tumors (Figs. 3 and 4). As has been noted previously (16), such clonal mutations indicate that the mutation either provided a selective growth advantage to the cell or occurred coincidentally with another mutation that was responsible for the clonal expansion. Such a coincidence is unlikely, because point mutations are generally considered rare events. In one study of colorectal carcinomas, for example, no point mutations were observed at over 10,000 restriction endonuclease recognition sites encompassing more than 40,000 bp (16). Thus, the finding of independent clonal mutations within the 1179 bp of the p53 coding sequences in two different tumors probably did not represent random events unrelated to tumorigenesis. The position of these mutations in a highly conserved region of the protein also suggested a functional change, as noted below.

Although the gene encoding p53 has been considered an oncogene (8), several studies have suggested that the normal p53 gene might have suppressor activity. First, it has recently been shown that normal p53 genes do not function as oncogenes during in vitro transformation; only mutated forms have this capacity (17-19). The mutations in colorectal tumors Cx1 and Cx3 both occurred in highly conserved positions of the p53 gene. Mutations in this region have been shown to confer in vitro oncogenicity to murine p53 genes (17-19). Such mutant p53 gene products can form complexes with normal p53 proteins, perhaps inhibiting their function (19). Second, the only other candidate tumor suppressor gene so far identified is the retinoblastoma susceptibility (Rb) gene (20). Both the Rb and p53 gene products interact with the large T-antigen of SV40 (21, 22), and it has been suggested that the large T-antigen gene functions as an onco-

gene because the binding of its gene product inactivates the suppressor function of the Rb protein or p53 (or both) (22, 23). Similarly, the adenovirus E1A and E1B gene products may contribute to viral oncogenicity by binding the Rb protein and p53, respectively (24). Third, p53 genes are often inactivated through proviral integration in Friend virus-induced mouse leukemias (25, 26). Fourth, rearrangements of the p53 gene occur in the human leukemia cell line HL60 and in some osteosarcomas, and no p53 gene product is detectable in HL60 cells (27).

On the basis of these observations, it is reasonable to speculate that the normal p53 gene interacts with other macromolecules (DNA or proteins) to result in suppression of the neoplastic growth of colorectal epithelial cells. This suppression is relieved if p53 expression is extinguished or if p53 mutations prevent the normal interaction of p53 with other cell constituents. Mutant p53 gene products might compete with normal p53 proteins and so act in a "dominant negative" fashion (17-19, 23, 25, 28), but a more pronounced effect of a mutated p53 gene might be realized when the normal allele is lost from the tumor. This hypothesis could explain why allelic deletions on chromosome 17p are so common in colorectal tumors, and would be consistent with postulated mechanisms of tumor progression (mutation of p53 at one step and loss of the normal p53 allele at another step near the adenoma-carcinoma transition point).

Another possibility concerns the relation between activated RAS and p53. As mentioned above, mutant mouse p53 genes can cooperate with mutant RAS to transform primary rodent embryo cells in vitro (29). Colorectal tumors are one of the few types of human neoplasms in which RAS mutations occur commonly (1, 2). The joint occurrence of p53 and RAS mutations in colorectal tumors would provide a provocative parallel with in vitro systems.

Although there is much to be learned, several of the issues raised here are experimentally approachable. Further sequencing studies as well as experiments to determine the biologic effect of wild-type and mutant human p53 genes on colorectal tumor cells should prove informative.

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Expression of a Cloned Rat Brain Potassium Channel in *Xenopus* Oocytes

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Potassium channels are ubiquitous membrane proteins with essential roles in nervous tissue, but little is known about the relation between their function and their molecular structure. A complementary DNA library was made from rat hippocampus, and a complementary DNA clone (RBK-1) was isolated. The predicted sequence of the 495-amino acid protein is homologous to potassium channel proteins encoded by the *Shaker* locus of *Drosophila* and differs by only three amino acids from the expected product of a mouse clone MBK-1. Messenger RNA transcribed from RBK-1 in vitro directed the expression of potassium channels when it was injected into *Xenopus* oocytes. The potassium current through the expressed channels resembles both the transient (or A) and the delayed rectifier currents reported in mammalian neurons and is sensitive to both 4-aminopyridine and tetraethylammonium.

POTASSIUM CHANNELS ARE MEMBRANE proteins that are selectively permeable to K^+ ions (1). Some kinds of K^+ channels are opened by depolarization; outward K^+ currents limit the duration of single action potentials (delayed rectifier) or set the pattern of bursts of action potentials (transient or A current). Other K^+ channels are opened or closed by second messengers to mediate the actions of synaptic transmitters (1, 2). We have determined some functional properties of a mammalian K^+ channel of known primary structure by expression in *Xenopus* oocytes of a cDNA clone isolated from rat brain.

A cDNA library made from rat hippocampus polyadenylated RNA was screened with two radiolabeled oligonucleotide probes (3). The sequences of the probes were based on conservation of amino acid sequences

between the *Shaker* A (4-6) and MBK-1 (7) predicted proteins. *Shaker* is a family of *Drosophila* mutants that expresses abnormal transient or A-type K^+ channels; at least four distinct proteins encoded by the *Shaker* locus form K^+ channels when expressed in *Xenopus* oocytes. MBK-1 is a clone isolated from a mouse brain cDNA library having a homologous nucleotide sequence, but MBK-1 has not been shown to direct the formation of functional channels. One of the rat brain clones (RBK-1) that gave positive hybridization with both probes was purified, and the nucleotide sequence of the 1.7-kb cDNA insert was determined (Fig. 1). The sequence contains one long open-reading frame that encodes a protein of 495 amino acids (molecular mass, 54.6 kD). The translation product predicted from RBK-1 is 69% homologous to the 453 residues that constitute the core common to the different proteins that could be formed from transcripts of *Shaker* cDNA. Significantly great-

er homology is found within the predicted membrane-spanning regions H1 to H6, and in the arginine-rich amphipathic helix (S4), which may represent the channel voltage sensor. The putative translation products of RBK-1 and MBK-1 differ by only three of their 495 amino acids, and there are no differences in the H1 to H6 or S4 regions; the sequence is identical to the expected product of a cDNA clone (RCK-2) isolated from rat cortex (8).

The RBK-1 sequence was subcloned into a plasmid expression vector (3), and the mRNA transcripts synthesized in vitro were injected into *Xenopus* oocytes (9). Voltage-clamp recordings made 24 to 96 hours later showed that the oocytes that had been injected produced large outward currents in response to depolarizing voltage commands. The currents were dependent on the amount of mRNA injected (10) and were not seen in uninjected cells. Some of the properties of the current are shown in Fig. 2; from a holding potential of -80 mV the current was first observed with depolarizations to -50 mV and was maximally activated at 0 or $+10$ mV.

The current resulted from the movement of K^+ ions, as shown by measurement of its reversal potential (11) in different K^+ ion concentrations (Fig. 3). The reversal potential (E_{rev}) was linearly related to the extracellular potassium concentration ($[K]_o$) by $E_{rev} = m \log ([K]_o/[K]_i)$ (the Nernst equation) where $[K]_i$, the intracellular potassium concentration, was assumed to be 110 mM. The slope of the relation (m) was 55 ± 2 mV per decade (SEM, $n = 5$ oocytes; theoretical value is $RT/F = 58$), providing convincing evidence that the channels expressed in the oocyte membrane are highly selective for K^+ ions.

We also determined whether the properties of the expressed channels were similar to those of the known classes of K^+ channels in mammalian neurons. The current developed (activated) within a few milliseconds of the applied depolarization (12), and the voltage dependence of activation of the K^+ conductance was well fit by a Boltzmann function centered at -30 mV (Fig. 2C). When the depolarizing pulse was terminated, the current subsided within a few milliseconds (12). Steady-state inactivation was studied by changing the holding potential for 5 s before applying the depolarizing command to $+10$ mV; the midpoint of the inactivation curve was at about -30 mV ($n = 3$). The current inactivated only partially during a maintained depolarization of up to 10 s in duration. The inactivation had a fast component (time constant, 50 to 100 ms) and a much slower component (time constant, 5 to 10 s), neither of which was strongly

APPENDIX C

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Mutations in the *p53* gene occur in diverse human tumour types

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THE *p53* gene has been a constant source of fascination since its discovery nearly a decade ago^{1,2}. Originally considered to be an oncogene, several convergent lines of research have indicated that the wild-type gene product actually functions as a tumour suppressor gene³⁻⁹. For example, expression of the neoplastic phenotype is inhibited, rather than promoted, when rat cells are transfected with the murine wild-type *p53* gene together with mutant *p53* genes and/or other oncogenes^{3,4}. Moreover, in human tumours, the short arm of chromosome 17 is often deleted (reviewed in ref. 10). In colorectal cancers, the smallest common region of deletion is centred at 17p13.1 (ref. 9); this region harbours the *p53* gene, and in two tumours examined in detail, the remaining (non-deleted) *p53* alleles were found to contain mutations⁹. This result was provocative because allelic deletion coupled with mutation of the remaining allele is a theoretical hallmark of tumour-suppressor genes¹¹. In the present report, we have attempted to determine the generality of this observation; that is, whether tumours with allelic deletions of chromosome 17p contain mutant *p53* genes in the allele that is retained. Our results suggest that (1) most tumours with such allelic deletions contain *p53* point mutations resulting in amino-acid substitutions, (2) such mutations are not confined to tumours with allelic deletion, but also occur in at least some tumours that have retained both parental 17p alleles, and (3) *p53* gene mutations are clustered in four 'hot-spots' which exactly coincide with the four most highly conserved regions of the gene. These results suggest that *p53* mutations play a role in the development of many common human malignancies.

To search for mutations, two approaches were used, both based on the polymerase chain reaction¹² (PCR) (Fig. 1). For tumour cell lines and for xenografts passaged in athymic nude mice, complementary DNA was generated from messenger RNA using oligo(dT) as a primer. A 1,300 base-pair (bp) fragment including the entire *p53* coding region was generated from the cDNA using PCR, and this fragment was cloned and sequenced

in its entirety. For primary tumours, sufficient RNA was often not available for the first approach, and PCR was used to generate a 2.9-kilobase (kb) fragment from tumour DNA. This was the longest fragment that we could reproducibly amplify from the *p53* locus, and included all of the exons found to contain mutations through the first approach.

Using these approaches, we analysed *p53* sequences of tumours derived from the breast, lung, brain, colon or mesenchyme. Tumours of these types have been previously shown to exhibit frequent deletions of chromosome 17p when studied by restriction-fragment length polymorphism (RFLP) methods¹⁰. To test for allelic deletions, tumour DNA samples were digested with *HinfI* and, following Southern transfer, hybridized sequentially to two probes (p144D6 (ref. 13) and pYNZ22.1 (ref. 14)) detecting variable-number tandem-repeat ('VNTR' or 'minisatellite') sequences. DNA samples from normal tissues exhibited two alleles with at least one of these probes in 29 of 31 different individuals tested. Because of this high degree of polymorphism, allelic loss could be assessed with greater than 95% certainty in cell lines and xenografts, even when corresponding normal tissue was not available for comparison.

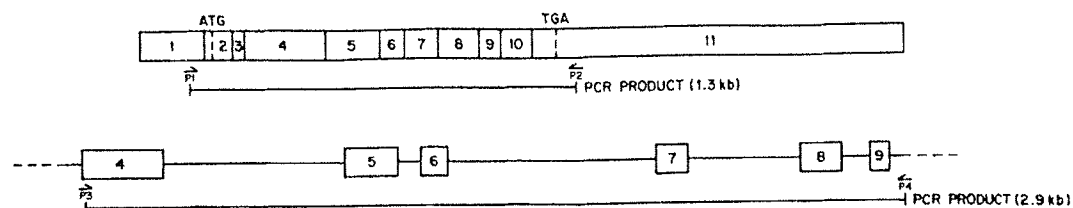
Nineteen tumours with allelic deletions of chromosome 17p were selected for sequence analysis. Thirteen of the tumours were found to contain a single missense mutation; two tumours each contained two missense mutations; one tumour contained a frame-shift mutation at codon 293; and no mutation was detected in three tumours (Table 1). The PCR reaction is known to be associated with a relatively high rate of base misincorporation¹², and we confirmed this observation by noting several sequence variants (13 out of 34,000 bp sequenced) in individual clones that were not reproducibly present in other PCR reactions from the same tumour sample. All of the mutations listed in Table 1 were confirmed by performing a second PCR reaction and re-sequencing the products *en masse* as described in the legend to Fig. 2.

Two observations indicated that the nucleotide substitutions described in Table 1 represented somatic mutations. First, none of these presumptive mutations have been observed in the sequences of human *p53* genes derived from normal cells, SV40-transformed fibroblasts, or lymphoblastoid cell lines (ref. 15, and references therein). Second, in six cases (tumours 2, 3, 9, 12, 13; 16), normal tissue from the patients whose tumours are described in Table 1 were available for study. To test for the presence of the presumptive mutations in the germline of these patients, a strategy was devised that used both PCR and cloning. Although direct sequencing of PCR products has been shown to be possible by several methods, we found that none of the published methods could be reproducibly applied to all parts of the *p53* coding region. To circumvent this difficulty, we cloned the PCR products into a phagemid vector and used the DNA pooled from 10³ to 10⁴ independent phage clones as a template for DNA sequencing (see legend to Fig. 2). This procedure resulted in sequence data quality as high as that produced using individual plasmid DNA clones as templates, and was used to demonstrate that in each of the six cases noted above, the mutations in the tumour DNA were not present in the germline of the patient (examples in Fig. 2).

The data described above indicated that most tumours with one 17p allele contained a mutation of the *p53* gene in the remaining allele. To begin to assess the status of tumours that had not lost a 17p allele, we examined cDNA clones from three such tumours. In each case, two cDNA clones derived from PCR products, generated as described in the legend to Fig. 1, were sequenced. In one case (tumour 11), both clones contained a single point mutation at codon 134 (Table 1). In the second case (tumour 16), one clone contained a point mutation at codon 281 and one clone was wild-type. In the third case (tumour 17), both clones were wild-type. To assess the relative expression levels of the mutant alleles, the sequencing strategy described

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FIG. 1 Strategies for amplification of *p53* gene sequences. Messenger RNA was used to generate a cDNA template for a polymerase chain reaction (PCR) using primers P1 and P2 (top). The PCR product was 1.3 kb long and included the entire coding region.



Alternatively, total genomic DNA was used in a PCR reaction using primers P3 and P4. The PCR product was 2.9 kb long and included exons 4–9 (bottom). The numbered boxes indicate exons and the vertical dotted lines indicate the start (ATG) and stop (TGA) codons respectively.

METHODS. RNA was purified using guanidium isothiocyanate and mRNA selected by binding to Messenger Affinity Paper (Amersham). Complementary DNA was synthesized from 500–750 ng of mRNA using oligo(dT) as a primer. The oligo(dT) primer was removed by isopropanol precipitation; 10 µg of transfer RNA and sodium perchlorate (to a final aqueous concentration of 0.5 M) were added to the reaction, and this was followed by addition of 1/2 volume of isopropanol²⁷. The cDNA was pelleted by centrifugation for 15 min at room temperature and used in a 50-µl PCR reaction consisting

of 35 cycles of 93 °C (1 min), 58 °C (1 min), and 70 °C (2 min). Genomic DNA (2 µg) was used in a 200-µl PCR reaction consisting of 30 cycles at 95 °C (1 min), 58 °C (1 min), and 70 °C (4 min). PCR reactions contained magnesium chloride at a final concentration of 2 mM. The primers used were P1, 5'-GGAATTCACGACGGTGACACG-3'; P2, 5'-GGAATTCAAAATGGCAGGGG-AGGG-3'; P3, 5'-GTAGGAATTCGTCCCAAGCAATGGATGAT-3'; P4, 5'-CATCGA-ATTCTGGAACCTTCCACTTGAT-3'. All primers had extraneous nucleotides comprising *Eco*RI sites at their 5' ends to facilitate cloning. The PCR products were digested with *Eco*RI, fractionated by electrophoresis, and following purification from agarose, ligated to *Eco*RI digested Bluescript vectors (Stratagene). Individual clones were sequenced with primers derived from the *p53* coding and intron sequences¹⁹ using T7 polymerase.

in the legend to Fig. 2 was used using cDNA from tumour mRNA as a template. In tumour 11, only the mutant allele was expressed (data not shown); in tumour 16, the mutant and wild-type alleles were expressed at approximately equal levels (Fig. 2, panel 4).

The fact that three (tumours 5, 8 and 10) of the 19 tumours with single chromosome 17p alleles did not contain *p53* gene mutations has potentially important practical and conceptual implications. At least three explanations could account for this result. First, it is possible that mutations existed in these three tumours but were outside the region sequenced. Such mutations could affect the transcription of *p53* mRNA, its stability or translational capacity. Experiments to test this possibility are in progress, but it is notable that although tumour 5 expressed *p53* mRNA, no *p53* protein could be detected on western blots (S. E. Kern and J.M.N., unpublished data). Alternatively, it is possible that a second tumour suppressor gene on chromosome 17p exists and was the target of allelic deletion in some tumours. Finally, the possibility that some allelic deletions represent 'nonselected losses', coincidentally occurring with other independent mutations elsewhere in the genome, cannot be excluded.

Neurofibrosarcomas are tumours that predominantly occur in patients with neurofibromatosis type I. This syndrome can be inherited in an autosomal dominant fashion, and the gene responsible for it (*NF*) has been mapped to chromosome 17q near the centromere¹⁶. Interestingly, chromosome 17 sequences are lost from neurofibrosarcomas from these patients, but the region of deletion sometimes includes the short arm and does not include the region of 17q harbouring the *NF* gene (T. G. and B. Seizinger, unpublished data). The result shown in Table 1 (tumour 12) suggests that a target of allelic deletion, in at least one neurofibrosarcoma, was *p53*.

Altogether, 20 point mutations (19 missense, 1 frameshift) were identified in the present study. These are mapped in Fig. 4, together with the two human *p53* gene missense mutations previously described⁹. Several features are notable. Although the sample size is limited, the mutations tended to be clustered in four hotspots which accounted for 86% of the 21 missense mutations (five mutations in region A, codons 132–143; five mutations in region B, codons 174–179; three mutations in region C, codons 236–248; five mutations in region D, codons 272–281). There have been two missense mutations identified in murine tumour cells, both in the carcinogen-induced fibrosarcoma cell line Meth A: one allele contained a mutation in region A, and the other contained one mutation in region C and one mutation in region D^{17,18}. Interestingly, the four hotspots for *in vivo* mutation coincided exactly with the four most highly conserved

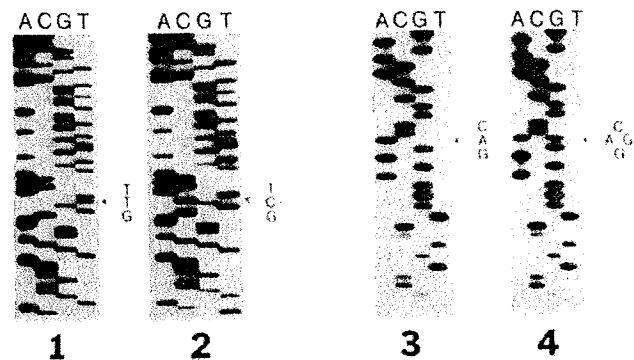


FIG. 2 Examples of sequencing reactions demonstrating *p53* gene mutations. The templates used for the sequencing reactions shown in panels 1–4 consisted of pools of greater than 10^3 clones generated from PCR products. Tumour 13 genomic DNA contained a mutation at codon 239 (antisense GCT, panel 2), instead of the wild-type sequence (GTT) found in the genomic DNA from normal lymphocytes from the same patient (panel 1). Panel 4 shows a sequencing reaction of pooled cDNA clones from tumour 16 showing that both wild-type codon 281 (GAC) and mutant codon 281 (GGC) were both expressed. Only the wild-type sequence (GAC) was found in pooled genomic DNA clones from normal lymphocytes of this patient (panel 3).

METHODS. PCR reactions were carried out as described in the legend to Fig. 1, and the reaction products digested with *Eco*RI. The entire reaction was ligated to 0.25 µg of λ ZAP phage vector arms (Stratagene) and packaged using 1/4 of a GIGA-Pack extract (Stratagene). *Escherichia coli* BB4 cells were then infected, and 10^3 – 10^4 phage clones plated on a 7-cm Petri dish. The λ ZAP vector contains the sequences for a phagemid into which the PCR inserts were cloned, and single-stranded DNA phage can be rescued from the λ phage clones using a helper phage²⁸. An overnight culture of XL-1 Blue cells (Stratagene) was grown in 0.4% maltose and resuspended in 1.5 volumes of 10 mM magnesium sulphate. Phages were eluted from the 7-cm dish in 5 ml phage-dilution buffer (100 mM sodium chloride, 10 mM magnesium sulphate, 20 mM Tris, pH 7.5, 0.02% gelatin) for 2 h at room temperature with gentle agitation. Fifty microlitres of eluate was used to infect 200 µl of XL-1 Blue cells (Stratagene) in the presence of 1 µl helper phage R408 (10^{11} P.F.U. per ml). After 15 min at 37 °C, 5 ml of 2×YT broth was added and the culture shaken for 3 h at 37 °C, then heated to 70 °C for 20 min. Cell debris was pelleted at 3,000g for 5 min, and 10 µl supernatant, containing single-stranded DNA phage, was used to infect 200 µl of XL-1 Blue cells, prepared as described above. After 15 min at 37 °C, 100 µl of the mixture (containing over 10^4 clones determined by titration on XL-1 Blue cells) was inoculated into 50 ml L-broth and shaken overnight at 37 °C. Double-stranded DNA was isolated by alkaline lysis and sequenced as described in the legend to Fig. 1. The primer used for sequencing in panels 1 and 2 was 5'-GAGGCAAGCAGAGGCTGG-3'. The primer used for sequencing in panels 3 and 4 was 5'-TGGAATCTACTGGGACG-3'.

TABLE 1 *p53* gene mutations in human tumours

Tumour	Tumour name	Tumour type*	Tumour cells tested†	Number of 17p alleles‡	Codon	Mutation Nucleotide	Amino acid
1	D263	Brain	B, X	1	175	CGC → CAC	Arg → His
2	D274	Brain	X	1	273	CGT → TGT	Arg → Cys
3	D303	Brain	B, X	1	216	GTG → ATG	Val → Met
4	D317	Brain	B, X	1	272	GTG → ATG	Val → Met
5	D247	Brain	C	1		None detected	
6	MDA 468	Breast	C	1	273	CGT → CAT	Arg → His
7	T47D	Breast	C	1	194	CTT → TTT	Leu → Phe
8	BT123	Breast	B	1		None detected	
9	1012	Lung	B	1	293	Deleted a G	Frameshift
10	5855	Lung	B	1		None detected	
11	H231	Lung	C	2	134	TTT → TTA	Phe → Leu
12	88-3/14	NFS	B, C	1	179	CAT → TAT	His → Tyr
13	C × 4A	Colon	B, X	1	239	AAC → AGC	Asn → Ser
14	C × 5A	Colon	X	1	248	CGG → TGG	Arg → Trp
15	C × 6A	Colon	X	1	132	AAG → AAC	Lys → Asn
					133	ATG → TTG	Met → Leu
16	C × 7A	Colon	B, X	2	281	GAC → GGC	Asp → Gly
17	C × 19A	Colon	X	2		None detected	
18	C × 20A	Colon	B, X	1	175	CGC → CAC	Arg → His
19	C × 22A	Colon	X	1	175	CGC → CAC	Arg → His
20	C × 26A	Colon	X	1	141	TGC → TAC	Cys → Tyr
21	SW480	Colon	C	1	273	CGT → CAT	Arg → His
					309	CCC → TCC	Pro → Ser
22	SW837	Colon	C	1	248	CGG → TGG	Arg → Trp

* The brain tumours were glioblastoma multiforme; the colon and breast tumours were adenocarcinomas, the NFS tumour was a neurofibrosarcoma developing in a patient with type-I neurofibromatosis; H231 was a small cell carcinoma of the lung, and the other two lung tumours were non-small-cell carcinomas.

† B, Tumour biopsy; C, cell line passaged *in vitro*; X, xenograft derived from biopsy, passaged in athymic nude mice. Whenever two sources of tumour cells are listed, both contained the indicated mutation.

‡ The number of alleles was determined by RFLP analysis, as described in the text.

regions of the *p53* gene, previously identified by Soussi *et al*¹⁹. Of the 41 amino acids contained within regions A–D, 93% are identical in the wild-type *p53* genes of amphibian, avian and mammalian species, compared to a conservation of only 51–57% over the entire *p53* coding sequence. The clustering of mutations and evolutionary conservation of regions A–D suggest that they play a particularly important role in mediating the normal function of the *p53* gene product.

Previous cytogenetic and RFLP studies have shown that allelic deletions of chromosome 17p occur in at least 60% of tumours of the colon, breast, lung, ovaries, cervix, adrenal cortex, bone and bladder, and in at least 30% of brain tumours^{10,20–23}. These tumour types account for most of the neoplasms occurring in humans. Our data suggest that the great majority of tumours with 17p allelic deletions contain mutant *p53* genes, and similar mutations occur in at least some tumours without 17p allelic deletions.

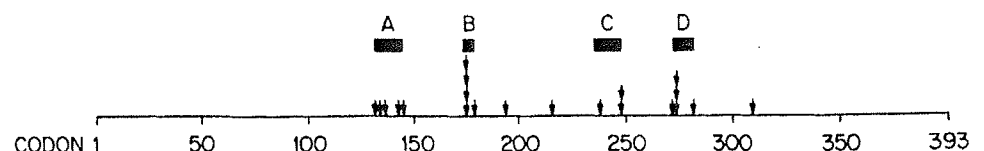
These data lead to the following hypothesis. Mutations in the *p53* gene occur during the process of tumorigenesis, and through a dominant negative effect^{3–5,9,24}, cause tumour progression. The dominant negative effect may be mediated by binding of the mutant *p53* product to the wild-type product, creating an inactive oligomeric complex^{18,25}. Because wild-type products remain in the cell, however, a further loss of growth control can be exerted when the wild-type allele is deleted, leaving the cell with only

a mutant allele. The first example of an intermediate step in this scheme is provided by tumour 16, which had not lost a chromosome 17p allele, but had developed a *p53* gene mutation and expressed both the wild-type and mutant alleles (Table 1 and Fig. 2). We imagine that tumour 16, had it not been surgically removed, would have eventually lost the wild-type *p53* gene through allelic deletion. Indeed, it has been shown that the loss of a chromosome 17p allele is significantly associated with tumour progression in the human host²⁶. Although this model requires much further study before it can be verified, it is now supported by several lines of research, including the demonstration of mutant *p53* genes in most tumours with 17p allelic losses. □

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FIG. 3 Summary of *p53* point mutations in human cancer. Each of the missense mutations listed in Table 1 is indicated with an arrow. In addition, the two point mutations described previously⁹ in human cancers (at codons 143 and 175) are also included. The four regions containing most (86%) of the mutations are indicated by the black bars marked A–D.



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Putative transcription activator with alternative isoforms encoded by human *ZFX* gene

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THE *ZFY* gene in the sex-determining region of the human Y chromosome encodes a protein with 13 zinc fingers, and may determine whether an embryo develops as a male or female¹. *ZFX*, a related gene on the human X chromosome, may also function in sex determination; it encodes a protein with a very similar zinc-finger domain and escapes X inactivation^{1,2}. *ZFY* and *ZFX* diverged from a common ancestral gene before the radiation of placental mammals, and retain a similar genomic organization². Analysis of complementary DNAs from the mouse Y-chromosomal homologues of *ZFY* indicates that these genes encode probable transcription activators^{3,4}. Here, we report that *ZFX* encodes a protein composed of a highly acidic amino-terminal domain, a basic putative nuclear-localization signal, and a carboxy-terminal zinc-finger domain. This combination of features, also found in the *ZFY* gene product, is typical of transcription activators. Alternative splicing generates *ZFX* transcripts encoding isoforms of 575 and 804 amino acids. These *ZFX* protein isoforms differ in the length of their acidic domains and may be functionally distinct.

ZFX is transcribed in all human cells analysed². We cloned *ZFX* cDNAs from a male lymphoblastoid cell line. Analysis by restriction mapping and hybridization with genomic probes revealed three distinct types of cDNAs. Whereas two types were represented by a single clone each (cDNAs 1 and 3), a third type was represented by three clones (cDNAs 2, 4 and 5).

We determined the nucleotide sequence of one cDNA of each type (Fig. 1). Comparison of cDNAs 1, 2, and 3 showed that alternative splicing and polyadenylation had produced structurally distinct 5' untranslated, coding, and 3' untranslated regions (Fig. 2a). Differential splicing involved an invariant donor site (nucleotide -378) and alternative acceptor sites (at nucleotides +647, -28 and -377, in cDNAs 1, 2 and 3, respectively). Each cDNA contains a single long open reading frame (ORF), and in each case the first ATG occurs in a sequence context favourable for initiation of translation⁵. Complementary

FIG. 1 Composite nucleotide sequence of *ZFX* cDNAs 1, 2 and 3 (Fig. 2a) and predicted amino-acid sequences. Numbering of nucleotides and amino acids is with reference to the first in-frame ATG codon in cDNAs 2 and 3. Known splice sites are indicated by upward arrows. Alternative starts of poly(A) tails are indicated by downward arrows. Complementary DNA 1 begins at nucleotide -527, uses a splice donor site at -378 and an acceptor site at +647; a tail of 20 adenosines follows nucleotide 2,585. Complementary DNA 2 starts at nucleotide -426, uses the same donor, but a different acceptor at -28; a tail of 46 adenosine residues follows nucleotide 5,450. Complementary DNA 3 starts at nucleotide -426, uses the same donor, but yet another different acceptor at -377; a tail of 26 adenosines follows nucleotide 3,298. Complementary DNAs 2 and 3 contain the same ORF with the first in-frame ATG codon at nucleotide 1; the predicted protein of 804 amino acids is composed of an acidic N-terminal domain, a small basic domain (residues 391-406, boxed), and 13 zinc fingers (cysteines of the Cys-X-X-Cys repeats are circled). Complementary DNA 1 has a shorter ORF with the first in-frame ATG codon at nucleotide +688, encoding a protein of 575 residues. The 3' UTR of cDNA 2 contains an *Alu* sequence¹⁶ (nucleotides +3,959 to +4,250, boxed). An AATAAA polyadenylation signal¹⁷ occurs 20 nucleotides 5' of the poly(A) tail of cDNA 2. Similar sequences are present 5' of the poly(A) tails in cDNAs 1 and 3. METHODS. Complementary DNA libraries were prepared³ using poly(A)⁺ RNA prepared² from human male lymphoblastoid cell line WHT1659. Five million recombinant phages from unamplified libraries were screened using plasmids pDP1007, pDP1041 and pDP1006 (*ZFY* genomic fragments which cross-hybridize to *ZFX*)^{1,2} as probes. Complementary DNA inserts of seven phages were subcloned into Bluescript vectors (Stratagene). The full DNA sequences of three inserts and partial sequences of the other four inserts were determined². As judged by comparison with genomic sequences, five cDNAs originated from *ZFX* and two from *ZFY*.

DNAs 2 and 3, with identical ORFs, encode a protein of 804 amino acids (*ZFX*⁸⁰⁴), whereas cDNA 1 encodes an isoform of 575 amino acids (*ZFX*⁵⁷⁵). (Alternatively, with cDNA 1, translation initiation at a second ATG, whose context is highly favourable, would result in production of an isoform of 573 amino acids.) *In vitro* transcription and translation of cDNAs 3 and 1 yielded the predicted full-length and truncated proteins, respectively (data not shown). Both *ZFX* protein isoforms contain three domains—an N-terminal acidic portion (25% aspartic and glutamic acid), a small basic domain, and a C-terminal run of 13 zinc fingers, each with two cysteines and two histidines (Cys-Cys/His-His zinc fingers). The isoforms differ in that the acidic domain of *ZFX*⁵⁷⁵ is half that of *ZFX*⁸⁰⁴.

Comparison of *ZFX* cDNAs with the genomic locus by restriction mapping and oligonucleotide hybridization gave an overview of the intron-exon organization (Fig. 2a). The C-terminal zinc-finger domain and 3' untranslated region (UTR) are encoded by a single exon, whereas the N-terminal acidic domain is encoded by a minimum of four exons for *ZFX*⁵⁷⁵ (cDNA 1) and a minimum of six exons for *ZFX*⁸⁰⁴ (cDNAs 2 and 3). The cDNAs span 67 kilobases (kb) in the genome.

Of the three types of cDNAs, type 2, encoding *ZFX*⁸⁰⁴, seems to be most representative of the 6.3- and 8-kb transcripts observed on northern blots². First, the coding exon defined by oligonucleotides '150' and '637' is present in cDNAs 2 and 3 but not in cDNA 1 (Fig. 2a). Northern analysis using the corresponding genomic DNA fragment revealed that this exon is present in the 6.3- and 8-kb transcripts (not shown). Second, northern analysis indicated that the polyadenylation site used in cDNA 2 corresponds, at least roughly, to the 3' end of the main *ZFX* transcripts (data not shown). Clone 2, which is 5.6 kb long, could represent a 6.3- or 8-kb transcript that is incomplete at the 5' end.

Transcripts corresponding to cDNA 1, encoding *ZFX*⁵⁷⁵, have not been detected by northern analysis. Using polymerase chain reaction (PCR) amplification, however, we confirmed the differential splicing predicted from cDNA analysis and crucial to the generation of *ZFX* protein isoforms (Fig. 2b). We designed splice-specific 5' primers spanning splices of the invariant donor site at nucleotide -378 to alternative acceptor sites at nucleotides +647, -28 and -377, as in cDNAs 1, 2 and 3, respectively. The